Effect of fluvastatin on apolipoprotein-defined lipoprotein subclasses in patients with chronic renal insufficiency

P Alaupovic[1], P-O Attman[2], C Knight-Gibson[1], H Mulec[2], L Weiss[2] and O Samuelsson[2]

[1]Lipid and Lipoprotein Laboratory, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA and [2]Department of Nephrology, Sahlgrenska University Hospital, Göteborg, Sweden

According to the concept of apolipoprotein (apo)-defined lipoproteins, apoA-I-containing lipoproteins consist of two subclasses referred to as lipoprotein A-I (LpA-I) and lipoprotein A-I:A-II (LpA-I:A-II), and apoB-containing lipoproteins of five subclasses, namely lipoprotein B (LpB), lipoprotein B:C (LpB:C), lipoprotein B:E (LpB:E), lipoprotein B:C:E (LpB:C:E), and lipoprotein A-II:B:C:D:E (LpA-II:B:C:D:E). The purpose of this study was to determine the levels of apoA-I- and apoB-containing lipoprotein subclasses before and after fluvastatin treatment of patients with chronic renal insufficiency. ApoA-I- and apoB-containing lipoprotein subclasses were measured in 15 patients with chronic renal failure and 15 asymptomatic subjects. The effect of fluvastatin on lipoprotein subclasses was determined in a randomized, double-blind, placebo-controlled, two-way, treatment period crossover study. Patients were administered fluvastatin 40 mg/day or placebo during 8 weeks in a randomized order. Patients were characterized by significantly higher levels of LpB ($P<0.001$), LpB:C ($P<0.001$), and LpB:E ($P<0.05$), and slightly higher levels of LpB:C:E and LpA-II:B:C:D:E than controls. The levels of LpA-I:A-II were significantly lower ($P<0.01$) in patients than controls. Fluvastatin treatment reduced all apoB-containing subclasses, but only the reduced level of LpB subclass was statistically significant ($P<0.02$). The levels of LpA-I and LpA-I:A-II were not affected. Fluvastatin treatment reduced and normalized LpB and LpB:E subclasses. Although slightly reduced, the levels of markedly atherogenic LpB:C subclass were not normalized. The potential role of LpB:C on the progression of coronary artery disease in chronic renal insufficiency remains to be determined in future studies.

Kidney International (2006) **69,** 1865–1871. doi:10.1038/sj.ki.5000327; published online 29 March 2006

KEYWORDS: chronic renal insufficiency; lipids; apolipoproteins; apolipoprotein-defined lipoprotein subclasses; immunoaffinity chromatography

Correspondence: *P Alaupovic, Lipid and Lipoprotein Laboratory, Oklahoma Medical Research Foundation, 755 Research Parkway, Suite 440, Oklahoma City, Oklahoma 73104, USA. E-mail: alaupovicp@omrf.ouhsc.edu*

Received 3 August 2005; revised 27 October 2005; accepted 18 November 2005; published online 29 March 2006

Traditionally, the lipoprotein abnormalities of chronic renal insufficiency have been characterized in terms of concentration changes in major lipoprotein density classes. Accordingly, renal dyslipoproteinemia has been broadly characterized by increased levels of very low- (VLDL) and intermediate-density (IDL) lipoproteins, unchanged or moderately raised levels of low-density (LDL) lipoproteins, and decreased levels of high-density lipoproteins (HDL).[1] However, studies on the apolipoprotein (apo) composition and immunochemical and kinetic properties have revealed a marked chemical and metabolic heterogeneity of major lipoprotein density classes, which has been accounted for by the occurrence of discrete lipoprotein subclasses characterized by similar density properties but specific qualitative apo composition.[2] These findings led to the introduction of an alternative classification system of lipoproteins based on apo composition rather than physical properties as the main criterion for their identification and differentiation.[2] This classification system recognizes two lipoprotein classes, one of which is characterized by apoA and the other by apoB as the major lipoprotein constituents. The former lipoprotein class consists of two major lipoprotein subclasses named according to their apo composition, lipoprotein A-I (LpA-I) and lipoprotein A-I:A-II (LpA-I:A-II), whereas the latter encompasses five major subclasses called lipoprotein B (LpB), lipoprotein B:E (LpB:E), lipoprotein B:C (LpB:C), lipoprotein B:C:E (LpB:C:E), and lipoprotein A-II:B:C:D:E (LpA-II:B:C:D:E).[2,3] Each of these lipoprotein subclasses is characterized not only by a unique apo composition but also by specific metabolic and functional properties.[2,3] Furthermore, it appears that apoB-containing lipoprotein subclasses may differ in their atherogenic capacities[3–5] and apoA-containing lipoproteins in their antiatherogenic potentials.[3,6–8]

The subfractionation and quantification of individual apo-defined subclasses required the use of specific immunologic methods such as immunoprecipitation[2,9,10] and/or immunoaffinity chromatography.[2,11] A stepwise immunoaffinity chromatographic procedure developed for separating and quantifying five apoB-containing lipoprotein subclasses has now been applied, for the first time, to the determination

of apo-defined lipoprotein subclass profiles in patients with chronic renal insufficiency and subjects with asymptomatic normolipidemia.

In our recent study on the modulation of renal dyslipoproteinemia by fluvastatin,[12] a synthetic hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, we have shown that this statin was quite effective in reducing and normalizing lipid and apo constituents of cholesterol-rich lipoproteins, but less effective in lowering those of triglyceride (TG)-rich apoB-containing lipoproteins. Thus, the purpose of this study was to determine by use of a stepwise immunoaffinity chromatography procedure both the lipoprotein concentration profile and the effect of fluvastatin on five apoB-containing lipoprotein subclasses in patients with moderately advanced renal insufficiency.

RESULTS

ApoB-containing lipoprotein subclasses

As shown in the initial report of the fluvastatin intervention trial,[12] patients with renal insufficiency had significantly higher concentrations of total cholesterol (TC), TG, VLDL-C, and LDL-C and lower levels of HDL-C than control subjects. Similarly, the concentrations of apoB, apoC-III, and apoE were significantly higher in patients than controls. The slightly lower levels of apoA-I in patients compared to those in controls were not statistically significant. In comparison with controls, patients had significantly higher levels of apoC-III bound to apoB-containing lipoproteins (apoC-III-heparin-manganese precipitate), but their slightly higher levels of apoC-III bound to apoA-containing lipoproteins (apoC-III-heparin-manganese supernate) did not reach statistical significance.

The measurement of discrete apoA-containing lipoprotein subclasses showed that patients with renal insufficiency had normal levels of putative antiatherogenic LpA-I particles, but significantly decreased levels of LpA-I:A-II particles compared to control subjects.[12] Thus, the lower levels of plasma apoA-I in chronic renal insufficiency were owing to a decrease in the concentration of LpA-I:A-II rather than LpA-I particles.

By using the newly developed immunoaffinity chromatographic procedure, it was possible to determine for the first time the concentrations of five discrete apoB-containing lipoprotein subclasses in renal patients and asymptomatic controls (Tables 1 and 2). The lipoprotein subclass profile of fasting normolipidemic subjects was characterized by cholesterol-enriched LpB and LpB:E particles as the main lipoprotein species of predominantly low-density properties accounting for 75% of the total apoB-containing lipoproteins. The intact and partially delipidized TG-rich LpB:C, LpB:C:E, and LpA-II:B:C:D:E particles of mainly very low- and intermediate-density characteristics accounted for 25% of the remaining apoB-containing lipoproteins. In patients with renal insufficiency, the levels of all five apoB-containing lipoprotein subclasses were found to be elevated in comparison with those of control subjects. The increased concentrations of plasma apoB and apoC-III reflected significantly increased levels of cholesterol-enriched LpB and TG-rich LpB:C particles rather than slightly, but non-significantly, elevated levels of LpB:E, LpB:C:E, and LpA-II:B:C:D:E particles.

Table 1 | Patient characteristics at study entry

Characteristics	Men	Women
Sex (n)	10	5
Age (year)	57.0±11.7	57.9±12.4
Weight (kg)	80.1±8.1	73.0±12.2
Height (cm)	180.5±4.8	169.8±9.0
Body mass index (kg/m2 body surface area)	24.6±2.9	25.2±2.8
Duration, chronic renal disease (year)	14.9±11.9	17.6±1.6
GFR (ml/min 1.73 m2 BSA)	33.9±8.9	20.8±1.2

BSA, bovine serum albumin; GFR, glomerular filtration rate. Values are expressed as number of mean±s.d.

Table 2 | ApoB-containing lipoprotein particles in plasma of patients with chronic renal failure before dialysis and normolipidemic asymptomatic controls

Lipoprotein particles	Chronic renal failure (mg/dl) n=15	Normolipidemic asymptomatic controls n=15	P-value*
ApoB	136.6±39.5	93.6±28.7	<0.001
ApoC-III	19.2±7.8	10.0±2.7	<0.001
LpB	82.1±29.6	55.9±20.8	<0.001
LpB:E	20.2±7.5	14.5±5.5	<0.05
LpB:C	20.3±10.1	10.4±3.7	<0.001
LpB:C:E	2.1±3.6	1.1±1.4	NS
LpA-II:B:C:D:E	11.9±5.6	11.2±4.0	NS

apo, apolipoprotein; Lp, lipoprotein; NS, non-significnt.
*Student's t-test.

Effect of fluvastatin on ApoB-containing lipoprotein subclasses

Renal patients randomized into placebo and fluvastatin treatment had comparable values for plasma lipids (Table 3), apo's (Table 4), and individual apoA- and apoB-containing lipoprotein subclasses at the start of each treatment period (Tables 3–5). TC (−18%) and LDL-C (−25.5%) were significantly reduced ($P=0.001$) by fluvastatin treatment. A slight reduction in the levels of TG (−13%) and VLDL-C (−11%) was not significant, whereas the concentration of HDL-C remained unchanged (Table 3).

Among plasma apo's, only the apoB (−18%) and apoE (−15%) were significantly reduced by fluvastatin (Table 4). Although plasma apoC-III levels remained unchanged, fluvastatin treatment influenced moderately the distribution of apoC-III between apoA- and apoB-containing lipoproteins. There was a slight reduction of apoC-III bound to apoB-containing lipoproteins (apoC-III-heparin-manganese precipitate) and a corresponding elevation of apoC-III bound to apoA-containing lipoproteins (apoC-III-heparin-manganese supernate); the resulting increase in the apoC-III-ratio (+18%) suggested a slightly enhanced catabolism of TG-rich

Table 3 | Plasma concentrations of lipids before and after treatment with placebo and fluvastatin

	(*n*=15)					
	Placebo (mg/dl)		Fluvastatin		Adjusted treatment effect (%)	*P*-value
	Baseline	8 weeks	Baseline	8 weeks		
TC	248.3±54.2	239.6±53.3	240.3±58.2	190.8±42.3	−18.0	0.001
VLDL-C	36.8±17.2	32.8±14.0	35.9±13.1	28.1±0.1	−11.0	>0.20
LDL-C	167.6±48.9	161.6±42.0	159.5±56.7	113.4±34.7	−25.5	0.001
HDL-C	39.9±7.5	38.4±6.6	42.3±6.9	41.5±9.2	1.9	>0.20
TG	225.5±137.1	209.9±136.2	213.8±108.6	169.8±108.3	−13.1	>0.20

HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride; VLDL-C, very low-density lipoprotein-cholesterol. Values are expressed as mean±s.d.

Table 4 | Plasma concentrations of apo's before and after treatment with placebo and fluvastatin

	(*n*=15)					
	Placebo (mg/dl)		Fluvastatin		Adjusted treatment effect (%)	*P*-value
	Baseline	8 weeks	Baseline	8 weeks		
ApoA-I	128.6±18.1	126.3±14.7	125.2±12.9	131.5±15.6	6	0.10
ApoB	141.0±37.6	140.0±38.6	135.4±38.0	109.7±32.0	−18	0.0001
ApoC-III	20.5±9.6	19.2±10.3	19.3±7.7	18.2±8.5	−0.2	>0.20
ApoE	11.7±4.7	11.0±3.8	11.7±3.8	9.2±2.4	−15	0.01
ApoC-III-HS	7.9±4.3	7.0±2.7	6.9±3.0	7.6±2.6	1	0.14
ApoC-III-HP	10.5±6.7	10.8±8.0	11.4±6.3	8.9±6.1	−20	0.07
ApoC-III-ratio	1.16±1.35	0.88±0.53	0.72±0.37	1.14±0.84	18	0.08

Apo, apolipoprotein; HP, heparin-manganese precipitate; HS, heparin-manganese supernate. Values expressed as mean±s.d.

Table 5 | Plasma concentrations of ApoA-I- and ApoB-containing lipoprotein subclasses before and after treatment with placebo and fluvastatin

	(*n*=15)					
	Placebo (mg/dl)		Fluvastatin		Adjusted treatment effect (%)	*P*-value
	Baseline	8 weeks	Baseline	8 weeks		
LpA-I	32.9±5.6	31.6±4.4	33.6±4.3	35.1±6.8	7	0.19
LpA-I:A-II	95.7±13.6	94.7±10.7	91.6±9.8	96.4±11.1	5	>0.20
LpB	85.4±25.7	81.7±30.6	81.0±30.1	64.4±25.4	−15	0.02
LpB:E	20.2±7.0	19.8±6.0	19.8±7.1	14.1±6.4	−26	0.16
LpB:C	19.3±10.1	21.0±1.01	20.0±8.3	17.6±9.3	−19	0.12
LpB:C:E	2.2±3.5	1.6±2.5	1.4±3.2	1.9±3.0	79	>0.20
LpA-II:B:C:D:E	13.4±3.9	13.5±6.0	15.4±5.8	11.8±5.3	−22	0.11

Apo, apolipoprotein; Lp, lipoprotein. Values expressed as mean±s.d.

lipoproteins. Fluvastatin treatment had no effect on apoA-I levels.

All four major apoB-containing lipoprotein subclasses were reduced by fluvastatin treatment, with the exception of low levels of LpB:C:E subclass (Table 5). However, only the reduced concentration of cholesterol-rich LpB subclass was statistically significant. A comparison between fluvastatin-treated patients (Table 5) and normolipidemic controls (Table 2) showed that fluvastatin not only reduced but almost normalized the cholesterol-enriched LpB and LpB:E subclasses and their characteristic constituents, including plasma TC, LDL-C, apoB, and apoE. In contrast, the levels of intact or partially delipidized LpB:C subclass and the corresponding constituents such as plasma TG, VLDL-C, apoC-III, and apoC-III-heparin-manganese precipitate were reduced, but not normalized; differences between normal and treatment values of these variables remained statistically significant ($P<0.01$).

Adverse events

Fluvastatin was generally well tolerated. As already presented for the whole cohort of 48 patients with chronic renal failure treated with fluvastatin,[12] there were 37 non-serious adverse events in 22 patients during fluvastatin treatment, and 49 non-serious adverse events in 28 patients during placebo therapy. The most common reports were gastro-intestinal symptoms of various types (diarrhea, dyspepsia, nausea, abdominal pain, and constipation) with no difference in frequencies between placebo and active treatments. All 15 patients selected for the measurement of apoA- and apoB-containing lipoprotein subclasses completed the study.

DISCUSSION

Results of this and previous studies[12–15] have shown that in renal insufficiency the profile of individual apoA-containing lipoprotein subclasses is characterized by normal levels of LpA-I and slightly to moderately reduced levels of LpA-I:A-II subclasses. If one considers that LpA-I subclass represents the antiatherogenic factor of apoA-containing lipoproteins or HDL, a moderate decrease in the levels of LpA-I:A-II, but not LpA-I, might be of little clinical significance.

The measurement of individual apoB-containing lipoprotein subclasses in the present study has indicated that significantly elevated concentrations of cholesterol-rich LpB and TG-rich LpB:C are the most characteristic abnormalities of dyslipoproteinemia of chronic renal failure. What might be a clinically significant aspect of this lipoprotein profile is the occurrence of relatively high levels of LpB and LpB:C subclasses considered to be the most atherogenic among the apoB-containing lipoproteins. As the main subclass of LDL, the LpB particles have been shown to have a significant atherogenic potential based indirectly on a number of lipid-lowering trials[16] and by their direct measurement and association with the progression of atherosclerotic lesions in the Monitored Atherosclerosis Regression Study.[17] Similarly, it was suggested in the Cholesterol Lowering Atherosclerotic Study[18] as well as in the Monitored Atherosclerosis Regression Study and Cholesterol and Recurrent Events ancillary studies[19] that intact or partially delipidized apoB-containing lipoproteins enriched with apoC-III may have atherogenic potential similar to that of LpB. The concentrations of LpB:C, but not LpB or LpB:C:E, were found to be significantly higher in predialytic subjects with chronic renal insufficiency than in corresponding controls.[20] In a recently reported ancillary Cholesterol and Recurrent Events study,[5] the measurement of LpB and LpB:C in the intermediate-density lipoprotein and LDL of subjects with type II diabetes provided the first opportunity to compare the predictive power of these two atherogenic subclasses. The results showed that LpB:C particles were a several-fold greater predictor of recurrent coronary events than LpB particles and that the relationship of LpB:C and coronary events was independent of lipid and apo risk factors. This important finding, suggesting that the LpB:C subclass may have a greater atherogenic potential than the LpB subclass, has provided additional evidence for the concept of the relative atherogenicity of individual apoB-containing lipoproteins.[2] Based on these findings, increased levels of both cholesterol-rich LpB and intact or partially delipidized TG-rich LpB:C subclasses ought to be considered as the main target of therapeutic intervention in patients with chronic renal failure.

To explore the effect of pharmacologic agents on the levels of LpB and LpB:C subclasses in patients with chronic renal failure, we have selected fluvastatin for the initial study, because this HMG-CoA reductase inhibitor has already been shown to be an efficient drug for lowering apoB-containing lipoproteins and plasma apo's C-III and E,[21–27] to have a good safety record in hyperlipidemic subjects,[24,26–32] favorable pharmacokinetic profile in patients with impaired renal function,[12] and an equally angiographic and clinical benefit, especially in non-renal patients with coronary artery disease.[31–34] As expected of a statin, fluvastatin reduced and, more importantly, normalized the levels of cholesterol-rich LpB and LpB:E subclasses, including their characteristic lipid and apo constituents (TC, LDL-C apoB, and apoE). In contrast, fluvastatin had very little effect in reducing and normalizing the concentration of atherogenic, TG-enriched LpB:C particles, or VLDL-C, TG, and apoC-III. There was practically no effect on the levels of TG-rich LpB:C:E and only a slight lowering effect on the levels of LpA-II:B:C:D:E. These findings confirm the well-established lowering effect of fluvastatin on cholesterol-rich apoB-containing lipoproteins in hypercholesterolemia,[21,27,30–32,35] combined hyperlipidemia,[36] type II diabetes,[28] and chronic renal failure.[12,37] A small insignificant lowering effect of fluvastatin on plasma levels of TGs and apoC-III, as reflected in practically unchanged concentrations of TG-rich apoB-containing subclasses presented in this study, is consistent with the results of a number of studies showing minimal[27,29–32,36,38–40] or moderate[24–26,28,34,41] lowering effect of fluvastatin on the levels of TGs and apoC-III. Although it appears that treatment with fluvastatin is more effective in reducing cholesterol-rich than TG-rich apoB-containing lipoprotein subclasses, it was shown in non-renal patients with mild to moderate cholesterol elevation[31,34] and in patients following successful percutaneous coronary intervention[41] that fluvastatin treatment reduced insignificantly in some cases[31] and significantly in other[34,41] the risk of major adverse cardiac events. While this effect may be attributed to lowering of the cholesterol-rich LpB and/or LpB:E subclasses, the lack of fluvastatin capacity to lower or normalize the levels of intact or partially delipidized TG-rich apoB-containing lipoprotein subclasses may diminish its overall antiatherosclerotic potential. The already observed contribution of complex TG-rich apoB-containing lipoprotein subclasses to the progression of renal dysfunction[42] in concert with the evidence for a significant role of hypertriglyceridemia in the development of endothelial dysfunction[43–45] and coronary heart disease[46] suggest strongly that increased levels of some TG-rich lipoproteins may also be a significant risk factor for the progression of coronary artery disease in renal insufficiency. It should be emphasized, however, that the relative contribution of cholesterol-rich and TG-rich apoB-containing lipoproteins to the progression of atherosclerosis in renal insufficiency remains to be determined in a clinical trial specifically designed to test this still unanswered question regarding the complex nature of renal dyslipoproteinemia. In this respect, it has recently been reported[47] that, despite a 42% decrease in the levels of LDL-cholesterol, atorvastatin had no significant effect on the incidence of cardiovascular and cerebrovascular events in patients with type II diabetes undergoing hemodialysis; it has not been established, however, to what extent increased levels of TG-rich lipoproteins might have contributed to the negative outcome of this trial.

In conclusion, this is the first study characterizing dyslipoproteinemia of chronic renal failure based on apo-defined apoB-containing lipoprotein subclasses. By using a three-step immunoaffinity chromatography, chronic renal failure was characterized by significantly increased levels of cholesterol-rich LpB and LpB:E and TG-rich LpB:C subclasses and moderately increased levels of TG-rich LpB:C:E and LpA-II:B:C:D:E subclasses in comparison with asymptomatic, normolipidemic controls.

Intervention with fluvastatin resulted in reduction and normalization of main constituents and number of cholesterol-rich LpB and LpB:E and TG-rich LpB:C:E and LpA-II:B:C:D:E; however, the levels of TG-rich LpB:C particles considered to be of substantial atherogenic capacity were slightly reduced, but not normalized. The potential role of LpB:C subclass in the initiation and progression of coronary atherosclerotic disease in chronic renal failure remains to be determined in future studies.

MATERIALS AND METHODS

Study group

Fifteen patients (10 men and 5 women) with chronic renal disease were included in the study and randomized to receive fluvastatin 40 mg once daily or placebo. Only non-nephrotic patients with primary chronic renal disease and moderate to advanced renal insufficiency were eligible for the study. Patients with secondary renal disease (such as diabetes mellitus), ongoing immunosuppressive treatment, ongoing estrogen therapy, and lipid-lowering therapy were excluded from this study.

All 15 patients completed fluvastatin and placebo treatments. Patients' mean age was 57.7 ± 11.7 years. The average glomerular filtration rate was 33.9 ± 8.9 ml/min/1.75 m^2 body surface area for men and 20.8 for women. The entry characteristics of the patient study group are presented in Table 1.

Control subjects were recruited among asymptomatic, normolipidemic Swedish subjects, employees of the Department of Nephrology, University of Göteborg, Sweden. The control population consisted of 15 subjects (10 men and 5 women) with mean age 49 ± 9 years.

Study design

The study was a randomized, double-blind, placebo-controlled, two-way, treatment period crossover study. The patients received fluvastatin 40 mg once daily or corresponding placebo in randomized order. Each treatment period was 8 weeks long and divided by an 8-week-long wash-out period.

Plasma lipids and apo's

Venous blood samples were drawn into ethylenediamine tetraacetate-containing vacutainer tubes after an overnight fast (8 h or more) with patients and controls in the recumbent position, and plasma samples were recovered by low-speed centrifugation for 10 min at 4°C. A preservative solution (0.13% ε-aminocaproic acid and 0.1% thiomerosal) was added (10 μl/ml) to all plasma samples, which were shipped in the fresh state by air express mail to the Lipid and Lipoprotein Laboratory, Oklahoma Medical Research Foundation, for measurements of lipids, apo's, and lipoprotein particles.

TC, TGs, and HDL-cholesterol (HDL-C) were determined by standardized enzymic procedures as described previously.[12] VLDL-cholesterol (VLDL-C) was assumed to equal one-fifth of the plasma TG concentration, and LDL-cholesterol (LDL-C) levels were calculated by the procedure of Friedewald *et al.*[48] TC, TG, and HDL-C were standardized through the lipid standardization program of the Centers of Disease Control and Prevention, Atlanta, GA, USA.

Measurements of apo's A-I, B, C-III, and E and the quantification of apoC-III bound to apoA- (HDL) and apoB- (VLDL + LDL) containing lipoproteins, performed on heparin-Mn^{++} supernates (apoC-III-heparin-manganese supernate) and heparin-Mn^{++} precipitates (apoC-III-heparin-manganese precipitate), were carried out as described previously.[12,49]

ApoA- and ApoB-containing lipoprotein subclasses

Plasma concentrations of LpA-I and LpA-I:A-II subclasses of high-density properties were determined by a differential electroimmunoassay on plates containing antisera to apo's A-I and A-II.[50]

Determination of plasma levels of five individual apoB-containing lipoprotein subclasses, LpB, LpB:E, LpB:C, LpB:C:E, and LpA-II:B:C:D:E, was performed by a modification of the originally described sequential immunoaffinity chromatography of apoB-containing lipoproteins.[11] Instead of a sequential separation, this modified procedure is based on a stepwise immunoaffinity chromatography using three immunosorbers with affinity-purified antibodies to apo's A-II, C-III, and E, respectively. The preparation of affinity-purified antibodies and the preparation and operation of immunosorbers have been described previously.[11,51,52] In the first step, aliquots of fresh plasma (0.15–0.3 ml) were applied to anti-apoA-II, anti-apoC-III, and anti-apoE immunosorbers and incubated overnight at room temperature (Figure 1). The elution of unretained (U) and retained (R) fractions and their concentration to a volume of 1.0 ± 0.5 ml was carried out as described previously.[11,51–53] After recording the final volumes, the apoB contents of unretained and retained fractions were quantified by electroimmunoassay.[49]

As shown in Figure 1, the retained fraction (R) from immunoaffinity chromatography of whole plasma on anti-apoA-II immunosorber contained LpA-II:B:C:D:E and the unretained fraction (U) contained the other four apoB-containing lipoproteins, including LpB, LpB:C, LpB:E, and LpB:C:E.

The retained fraction (R) from the anti-apoC-III immunosorber contained apoB-containing lipoprotein families characterized by the presence of apoC-III (LpB:C, LpB:C:E, and LpA-II:B:C:D:E) and the unretained fraction (U) contained LpB and LpB:E (Figure 1). Both the unretained and retained fractions were analyzed for their apoB contents. The unretained fraction was used for the separation of LpB and LpB:E.

The retained fraction (R) from the anti-apoE immunosorber included all apoB-containing lipoproteins in association with apoE (LpB:E, LpB:C:E, and LpA-II:B:C:D:E), whereas the unretained fraction (U) contained LpB and LpB:C (Figure 1). ApoB was determined in both fractions, and the unretained fraction was used for the separation of LpB and LpB:C.

The purpose of the second step in the separation procedure was to fractionate unretained fractions from anti-apoC-III and anti-apoE immunosorbers. The former unretained fraction (apoC-III-U) was applied to the anti-apoE immunosorber (Figure 1); the retained fraction contained LpB:E and the unretained fraction contained LpB. Both fractions were characterized by quantitative determination of apoB. Similarly, LpB and LpB:C were separated by affinity chromatography of the unretained fraction from anti-apoE

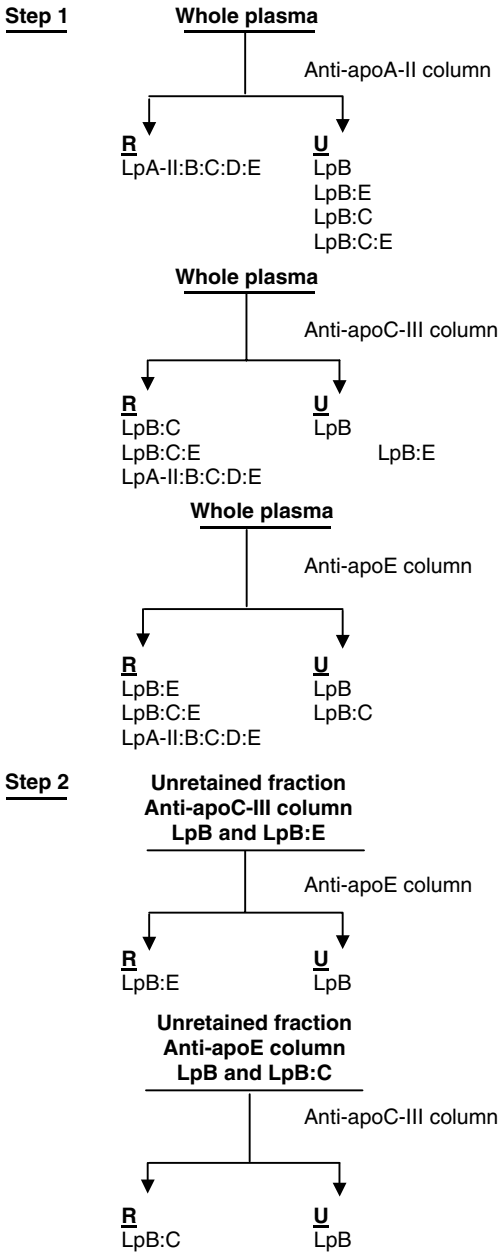

Figure 1 | Stepwise immunoaffinity chromatography for the separation and quantification of five apoB-containing lipoprotein subclasses.

immunosorber (apoE-U) (Figure 1). The retained fraction contained LpB:C and the unretained fraction contained LpB.

The concentrations of LpB, LpB:E, and LpB:C and LpA-II:B:C:D:E were determined and expressed on the basis of apoB concentration applied to appropriate immunosorbers and apoB recovered in the corresponding retained and unretained fractions using isolation schemes depicted in Figure 1. For example, the concentration of LpA-II:B:C:D:E particles was determined from the known content of plasma apoB applied to anti-apoA-II immunosorber and apoB contents recovered in retained and unretained fractions. If the recovery of apoB in retained and unretained fractions was lower than 100%, the recovered quantities of apoB were corrected to plasma apoB values; the acceptable recoveries of apoB ranged between 75 and 95%. Although LpB, LpB:E, LpB:C, and LpA-II:B:C:D:E families were determined directly in their isolated fractions (Figure 1), the levels of apoB in LpB:C:E particles were calculated as follows: LpB:C:E = apoER−(LpB:E + LpA-II:B:C:D:E).

The between-assay coefficient variation for LpA-II:B:C:D:E subclass was 9–10% (retained fraction from anti-apoA-II column). The between-assay coefficient of variation for LpB:C, LpB:C:E, and LpA-II:B:C:D:E (retained fraction from anti-apoC-III column) was 8–9% and that for LpB and LpB:E (unretained fraction from anti-apoC-III column) was 6–7%. The coefficient of variation for LpB:E, LpB:C:E, and LpA-II:B:C:D:E (retained fraction from anti-apoE column) was 6–7%, and that for LpB and LpB:C (unretained fraction from anti-apoE column) was 1–2%.

Other clinical variables

The glomerular filtration rate was determined as the plasma or renal clearance of ^{51}Cr-ethylenediamine tetraacetate.[54] A physical examination was performed and glomerular filtration rate was determined at randomization and at the end of the second treatment period. For safety reasons, blood pressure was measured and a routine clinical laboratory evaluation was performed at the start and end of each treatment period. Blood pressure was recorded after 5 min of supine rest.

Statistical methods

Standard statistics were used to illustrate the salient features of data. Outcome variables were measured before and after each treatment period. The percentage of change from baseline was calculated for all efficacy variables. These data were analyzed using a mixed-model analysis of variance with fixed effects for sequence, period, and treatment and a random effect for patient within sequence. Analyses were performed using Statistical Analyzing System version 6.12 (SAS Institute Inc., Cary, NC, USA). The study was approved by the Ethical Committee of the University of Göteborg (Sweden) under Study No. 223-96. All patients gave their written informed consent.

ACKNOWLEDGMENTS

We gratefully acknowledge the colleagues and staff of the following renal units participating in this study for their contributions: Department of Nephrology, Karlstad Hospital, Karlstad, Sweden; Department of Nephrology, Sahlgrenska Hospital, University of Göteborg, Sweden. We also gratefully acknowledge the staff of the Lipid and Lipoprotein Laboratory, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA, for their skillful handling of the lipoprotein analyses, and Ms. Kathy Hampton for her excellent secretarial assistance and preparation of the manuscript. This study was supported in part by a research grant from AstraZeneca, Mölndal, Sweden, and resources of the Oklahoma Medical Research Foundation, Oklahoma City, OK, USA.

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